Isolation and Characterization of Nonesterified 3-Hydroxy Acids in Milk

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ABSTRACT

Nonesterified 3-hydroxy acids of unknown origin, ranging in chain length from 6 to 16 carbons are identified in milk. Content of 3-hydroxydecanoic acid is estimated at 25 to 50 μg per liter of milk. It is proposed that the 3-hydroxy acids originate in milk as side products of fatty acid synthesis by way of the Malonyl CoA pathway rather than as fatty acid degradation products.

INTRODUCTION

The presence of hydroxy acids in the lipids of milk has been established for some time. Several workers (1, 3, 9) demonstrated 4- and 5-hydroxy acids in the triglycerides of milk fat while Morrison and Hay (6) identified 2-hydroxy acids in the sphingolipids. Jurriens and Oele (3) and Schwartz (8) reported that milk fat contains numerous unidentified hydroxy acids with the functional group in other than the 4- and 5-carbon positions. This study establishes the presence of a series of nonesterified 3-hydroxy acids in milk.

EXPERIMENTAL

Isolation of Free Fatty Acids (FFA)

Four hundred milliliters of raw herd milk were pasteurized at 62 C for 30 min within 5 h of milking, cooled to 5 C, and centrifuged at 59,000 × *g* for 60 min. The cream plug was removed from the centrifuge tubes with a spatula and the clear supernatant recovered by decantation.

The total cream plug was ground into sufficient silicic acid, previously washed with ethyl ether, to give a free flowing mixture. The mixture was acidified to approximately pH 2.0 (determined by pH paper) with 5.5 N H_2SO_4 and extracted for 5 h (eight solvent turnovers per hour) with ethyl ether in a Soxhlet Extractor. The ethyl ether extract was passed through a 5 g silicic acid column prepared as described by McCarthy and Duthie (5). The column was washed with 125 ml of ethyl ether, and the free fatty acids were eluted with 60 ml of ethyl ether containing 2.5% phosphoric acid (85%) followed by 100 ml ethyl ether. The effluent was extracted with two 25 ml portions of distilled water to remove the phosphoric acid and dried with sodium sulfate.

Two hundred milliliters of supernatant were acidified to pH 2.7 with 2.5 N HCl, and the sample including precipitated soluble casein was freeze dried. The dried sample was extracted 4 h with 200 ml of a 2:1 mixture of chloroform-methanol in a Soxhlet Extractor (ten solvent turnovers per hour). The methanol was removed from the solvent mixture by three successive extractions with 50 ml of a .9% aqueous KCl solution. The chloroform layer was dried with sodium sulfate, evaporated to dryness, and the residue was taken up in 150 ml of ethyl ether. The ether solution was extracted with 40 and 20 ml of 1 N NaOH. The combined NaOH extracts were acidified with 13 ml of concentrated HCl and extracted twice with a total of 120 ml of ethyl ether. The ether solution was dried with 100 g of sodium sulfate.

Derivatization and Isolation of 3-Hydroxy Acids

Ether solutions of the FFA were evaporated to near dryness under vacuum in a 250 ml round bottom flask. Twenty milliliters of methylene chloride were added immediately to the flask, and the sample was dried with sodium sulfate. The solvent, with an additional 5 ml of methylene chloride used to wash the sodium sulfate, was removed under a stream of nitrogen at 23 C in a 45 × 15 mm screw cap vial. As the last traces of methylene chloride evaporated, the residue immediately was taken up in 4 ml

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of anhydrous methanol containing 1% H_2SO_4. The solution was heated at 60 C for 45 min, and the methyl esters were recovered by extraction with 20 ml of methylene chloride following dilution of the methanol-H_2SO_4 solution with 25 ml of distilled water. The methylene chloride solution of the methyl esters was washed with 15 ml distilled water, dried with sodium sulfate, and evaporated to approximately 1 ml under a stream of nitrogen.

The methylene chloride solution of methyl esters was spotted on .5 mm thick precoated preparative Silica Gel G thin-layer chromatographic plates and developed to 15 cm with a hexane-ethyl ether-acetic acid solvent system (80 to 20 to 1). Following development, the plates were air dried for 5 min, and the area corresponding to authentic 3-hydroxy acid methyl esters (2 to 4 cm from origin) was removed from the plate. The esters were eluted from the Silica Gel G with 20 ml of methylene chloride. The methylene chloride was washed with 2 ml of 1 N NaOH to remove acetic acid, dried with sodium sulfate, and reduced in volume to .1 ml in a 35 × 12 mm screw cap vial.

The trimethylsilyl ethers (TMSi) were prepared by the addition of 4 μl of N-O-bis (trimethylsilyl)-acetamide to a .1 ml methylene chloride solution of the 3-hydroxy acid methyl ester fraction followed by heating for 5 min at 50 C.

Gas-Liquid Chromatography (GLC) and Mass Spectrometry (MS)

Studies by GLC-MS were by the LKB Combination Gas Chromatograph-Mass Spectrometer.[2] The methyl esters of the 3-hydroxy acids were chromatographed on a 1.22 M × .32 cm stainless steel column packed with 7.5% ethylene glycol adipate and 2% phosphoric acid on Anakrom ABS. The TMSi ethers were chromatographed with a 1.83 M × .32 cm stainless steel column packed with 1% Se 30 on 100 to 200 mesh acid washed and silaned Chromosorb W. In both chromatographic procedures, the column temperature was programmed from 75 to 195 C at 4 C per min. The flash heater and molecular separator were maintained at 210 C, and helium served as the carrier gas. Mass spectra were obtained at a constant accelerating voltage of 3.5 kv and electron energy of 70 ev.

[2] Reference to brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

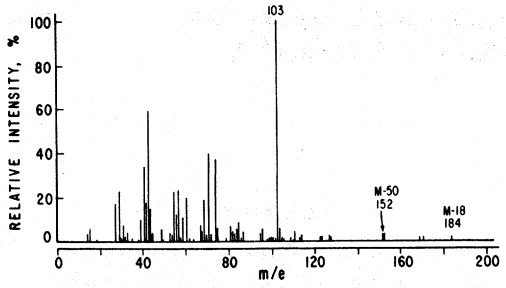

FIG. 1. Mass spectrum of methyl ester of 3-hydroxydecanoic acid isolated from milk.

Retention times of GLC and mass spectra of authentic C_{10}, C_{12}, and C_{14} 3-hydroxy acid methyl esters (Analabs, Inc., North Haven, Connecticut) and their TMSi ether derivatives were obtained for comparative purposes.

RESULTS AND DISCUSSION

Figure 1 presents the mass spectrum of the methyl ester of 3-hydroxydecanoic acid isolated from milk. The mass spectra of methyl esters of 3-hydroxy acids are characterized by an intense m/e 103 peak due to the fragment $^+CHOH\text{-}CH_2\text{-}CO_2CH_3$ formed by simple cleavage between carbon atoms 3 and 4 of the fatty acid residue. Although the higher mass end of the spectra of 3-hydroxy acid methyl esters is of low intensity and lacks a parent ion, the molecular weight can be inferred in samples of sufficient quantity by peaks at M-18 (loss of water) and M-50 (loss of water + methanol) (7).

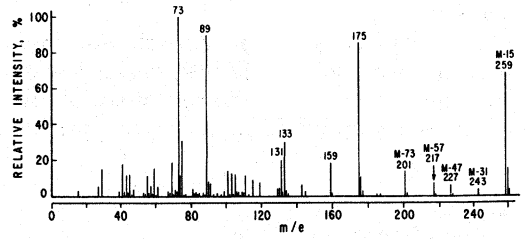

FIG. 2. Mass spectrum of TMSi ether of methylated 3-hydroxydecanoic acid isolated from milk.

TABLE 1. Nonesterified 3-hydroxy acids identified in the skim milk supernatant and cream fractions of centrifuged whole milk and the relative GLC peak heights of their methyl esters.

3-Hydroxy acid	Relative GLC peak heights[a]	
	Supernatant	Cream
C_6	.25	...
C_7[b]	trace	...
C_8	1.03	1.22
C_9	.08	trace
C_{10}	1.00	1.00
C_{12}	.41	.43
C_{14}	.06	.35
C_{16}	trace	1.83

[a]GLC conditions: Hewlett-Packard Model 5750 Gas Chromatograph; Column – 1.22 M × .32 cm, 7.5% EGA – 2% H_3PO_4 on Anakrom ABS; Helium pressure 30 psi, temperature programmed 75 to 195 C at 6 C/min; flash heater and detector at 250 C.

[b]Tentatively identified based on weak mass spectrum.

The TMSi ether derivatives of 3-hydroxy acid methyl esters (Fig. 2) are identified by peaks at m/e 73 (base peak), m/e 89, m/e 131, m/e 133, m/e 159, and m/e 175. The later peak represents 3, 4 cleavage resulting in the fragment $(CH_3)_3Si^+O{=}CH{-}CH_2{-}CO_2CH_3$ and is characteristic of TMSi ethers of 3-hydroxy acid methyl esters (2). Like the free 3-hydroxy esters, the TMSi ethers do not exhibit a parent peak. However, the molecular weight is inferred by a prominent M-15 peak and peaks of lesser intensity at M-31, M-47, M-57, and M-73 (2).

The 3-hydroxy acids identified as the TMSi ether of their methyl esters are in Table 1. In addition, the relative concentrations of the 3-hydroxy acids (based on GLC peak heights of their methyl esters) isolated by the procedures of this study are included. It is estimated on the basis of standard curves that 3-hydroxydecanoic acid is in milk at 25 to 50 μg per liter.

The origin of the 3-hydroxy acids identified in this study is unknown. One can speculate, however, that the acids originate as side products of fatty acid synthesis by way of the Malonyl CoA pathway rather than the result of fatty acid degradation. The apparent absence of 3-hydroxyoctadecanoic acid in milk lends support to this theory (4).

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